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Prevalence of vertically transmitted *Neospora caninum* amongst beef cattle in Phayao, Thailand

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Abstract

Neospora caninum the causative agent of neosporosis, is recognised as a significant trigger of abortion and productivity losses in cattle worldwide. Current information regarding to the prevalence of *N. caninum* in Thailand is limited due to the limitations of detection methods and the difficulty of recovering of viable parasite. Vertical transmission is the main route of *N. caninum* infection in cattle. Therefore, detection of *N. caninum* DNA in placental tissue could be a possible means of laboratory diagnosis of neosporosis in live animals, particularly in the context of transplacental transmission. The aim of this study was to investigate the prevalence of transplacentally transmitted *N. caninum* infection in female beef cattle in the Northern Thai province of Phayao by detection of *N. caninum* DNA in bovine placenta by PCR. A total of 96 bovine placentas were collected from 7 districts of Phayao. Our result indicated that overall PCR prevalence of *N. caninum* in cattle in this area was 36.5% varying from 16.7-50.0% between districts. The districts with the highest prevalence of infection were Muang (50.0%) and Mae Chai (44.7%). The proportion of *N. caninum* infection was quite high suggesting that newborn calves were at risk of congenital infection. This study provides a current snapshot of the status of bovine neosporosis in Phayao which could lead to the development of effective strategies for prevention and control this disease.

Keywords:*Neospora caninum*; Neosporosis; congenital infection; vertical transmission; bovine placenta

Introduction

Neospora caninum, an apicomplexan protozoa, is of significant importance in veterinary medicine; infecting a wide range species of economically important animals primarily in cattle. It is now recognised as one of the leading causes of infectious bovine abortion and responsible for the economic loss in cattle producing countries around the world [1]. It is generally accepted that vertical or trans-placental, transmission is the predominant route of bovine neosporosis transmission [2], allowing the parasite to transmit from generation to generations and maintain its presence in herds.

The presence of *N. caninum* in live animals is difficult to establish by traditional methods as the parasites are difficult to isolate and mostly in tissues non-amenable to sampling [3]. Therefore, epidemiological data on the occurrence of *N. caninum* infection in many parts of the world including Thailand is based upon seroprevalence. Previously in Thailand seroprevalence of *N. caninum* in cattle was shown to be around 5.5-70.0% of sampled animals across the country [4-8]. Although, serologic testing for *N. caninum* is a simple and non-invasive method suitable for confirming and screening for *N. caninum* infection [9], results can however vary dependent on the ELISA format used.

Pregnancy is associated with the changes in immune homeostasis, which can cause reactivation of the latent form of parasite to the actively replicating tachyzoite allowing the parasite to infect the foetus through transplacental transmission [3, 10]. Therefore, direct detection of whole parasites or their DNA could be possible in the pregnant animals. Polymerase chain reaction (PCR) already plays a significant part in the detection of *N. caninum* DNA in infected tissues of aborted foetuses and aborted materials producing high sensitivity and specificity compared to traditional methods [11, 12].

The objective of this study was to determine the prevalence, by molecular methods, of transplacentally transmitted *N. caninum* in naturally infected recently calved cattle raised in Phayao, Thailand, targeting their placenta.

Materials and Methods

Study area

Phayao province is located in the upper north of Thailand and is approximately 6,335 Km^2 in size. The province consists of 9 districts. According to the record of Department of Livestock Development (DLD), Thailand 2015, approximately 36,961 beef cattle exist in this province. The population of female beef cattle was 22,648 comprising 11,801 and 10,847 of heifers and cows respectively [13].

Study samples

The sample size (n = 96 animals) was calculated by the online n4Studies application for estimating the finite population proportion [14] by considering female cattle population in Phayao province (N =22,648), 46.9% seroprevalence of *N. caninum* in Northern Thailand [8], 95% confidence levels and 10% level of error.

Animals and specimen collections

A total of 96 bovine placentas were collected from full term pregnant cows within 6 h after calving. Cotyledon of each placenta was removed and stored at -20° C until molecular analysis. All studied cattle were beef cows aged between 2-8 years old raised by small farm holder (less than 30 cattle) across 9 districts in the Phayao province. Age and breed of each animal was recorded. The protocol for animal usage was approved by the University of Phayao Animal ethic Committee, protocol number UP-AE59-02-04-0001. Sampling was carried out between January and December 2016.

Detection of *N. caninum* DNA by PCR

Bovine placental DNA was extracted from 100 mg of cotyledon using QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The extracted DNA was stored at -20° C until used.

The PCR employed specific primers targeting NC-5 gene was carried as per Müller et al. [15] using NP6+ (5'-CTCGCCAGTCAACCTACGTCTTCT-3') and NP21+ (5'-CCCAGTGCGTCCAATCCTGTAAC-3'). In brief, 2 µl of DNA of each sample was subjected to a 25 µl PCR reaction containing 0.2 µM of each primer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$ and 2.5 U of Taq DNA polymerase in coral load PCR buffer, pH 8.7 (QIAGEN, Germany). For every PCR assay, DNA of *N. caninum* reference isolates (Nc-1 or Nc-2) provided by Veterinary Research and Development Center Upper Northeastern Region, were included as an internal control, negative controls, including reaction without DNA template and DNA of *Toxoplasma gondii*. The PCR program was pre-denaturing at 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 1 min and a final extension cycle at 72 °C for 5 min. The amplified PCR products were electrophoresed and visualised on 1.5% agarose gel pre-stained with Nancy-520 (Sigma-Aldrich, UK) under UV light. Gel images were recorded by Gel document. The expected PCR fragment for *N. caninum* was approximately 340 bp.

Sequencing

The PCR products from positive samples were randomly selected for sequencing to confirm specificity. The PCR fragments were purified by PureLink Quick Gel Extraction kit (Invitrogen, Carlsbad, CA). Sequencing was carried out by Macrogen (Korea) in both directions using both of NC6+ and NC21+ primers. The nucleotide sequences were blasted against available nucleotide sequences deposited in Genbank database using BlastN.

Statistical analysis

Descriptive statistics, including prevalence of *N. caninum* infection in each location and age group was determined. The prevalence of infection was estimated by the proportion of PCR positive results to the total number of specimen examined. Differences in the prevalence in each age group was analysed by Chi square test. P-value less than 0.05 were considered significant.

Results

A total of 96 bovine placentas were examined for the presence of *N. caninum* DNA. No abortion history was recorded in any of the studied animals. The numbers of samples collected from individual districts were as follows Mae Chai (38), Chiang Kham (13), Muang (12), Chiang Muan (12), Dok Kamtai (11), Pong (6) and Phu Sang (4).

In this study a conventional PCR targeting the Nc-5 region of *N. caninum* was used to determine the presence of *N. caninum* DNA in the bovine placenta. By this analysis, 35 positive samples were obtained. Only PCR amplicons of the expected size were obtained suggesting that the reaction was specific in our samples. While the PCR is non-quantitative differing intensities of amplicons was noted in each sample suggestive that varying amounts of template was present (Fig. 1, Lanes 2, 3, 4, 7, 8, 11). Selected positive PCR products (5) were confirmed by sequencing and revealed sequences had between 96-99% amongst each other and 97-99% similarity with the Nc-5 DNA sequence of *N. caninum* deposited in GenBank database.

In our 96 placental samples 35 were PCR positive corresponding to an overall prevalence of 36.5%. PCR positive placentas were collected from 7 districts including Muang, Mae Chai, Dok Kamtai, Chiang Kham, Chiang Muan, PhuSang and Pong districts (Fig. 2). The highest detection of the parasite was observed in placenta collected from Muang and Mae Chai

districts with the prevalence of 50.0% and 44.7% respectively, while the lowest prevalence was recorded in cows from Pong district (16.7%).

Cattle were aged 2-8 years at the time of sampling. They were categorised into 3 groups; 2-4 years, over 5 years and a group of unknown age. *N. caninum* was detected in bovine placenta samples from all age groups. There were 13 (31.7%) positive samples for female age between 2-4 years, 15 (45.5%) for age group above 5 years and 7 (31.8%) positive sample for unknown age group (Table 1). The statistical analysis showed that there was no significant difference between older and younger aged cows (P>0.05).

Discussion

In this study, we have determined the prevalence of *N. caninum* in naturally infected female beef cattle in Phayao province using PCR. Our results demonstrated that over 1/3 of placental samples test, 36.5%, contained *N. caninum* DNA, indicating that vertical transmission of this parasite in beef cattle was common throughout the province. Not all districts in the province were equal in the prevalence of infection which varied from 16.7-50.0%; this however was not statistically significant. The prevalence of *N. caninum* infection was greatest in Muang (50.0%) and Mae Chai (44.7%) district while the lowest prevalence was recorded in female cows from Pong district. Of the 9 districts, there was no prevalence result in either the Phu Kam Yao or Chun districts due to the limitation of specimen collected from these districts.

Most animals, 74/96, in this study were between 2-8 years old and infection was observed in all age groups. PCR positivity of *N. caninum* was slightly higher in the animals older than 5 years with 45.5% of positive placenta tested but overall age differences were not deemed statistically significant (p>0.05). Our finding is consistent with the prevalence of bovine neosporosis in northeastern Thailand [6] and other reports from southeast Asian countries such as Lao [16], Indonesia [17] and Philippines [18] confirming that no association between age of animals and infection by *N. caninum* which suggests that transplacental transmission is

probably more important. In contrast, other studies observed significant relationship between age of animal and seropositivity indicating the possibility that older animals are more likely to be infected by contacting the oocyst of *N. caninum* from environmental through horizontal transmission [19]. Our observation suggests that transplacental infection may play a major role in *N. caninum* infection in cattle in this area, although oocyst ingestion (postnatal transmission) cannot be ruled out.

Vertical transmission is the most important form of *N. caninum* transmission in cattle due to the range of clinical outcomes in pregnant animals. The detection of high prevalence rates of *N. caninum* infection in female cattle could suggests there is a high risk of transplacental transmission; however it should be noted that not all latently infected dams produce congenitally infected calves. However, calves born from these PCR positive cows are at risk of congenital infection and could be a potential source of parasite transmission in this area. Previous studies demonstrated that up to 95.2% of the new born calves delivered from an *N. caninum* seropositive heifer displayed a positive antibody response against *N. caninum* presumably as the parasite becomes reactivated in seropositive pregnant dams and is actively transferred to the foetus [3, 10]. As a consequence vertical transmission plays an important role in maintaining *N. caninum* by successive propagation of the parasite from one generation to the next.

Not all animals infected with *N. caninum* always abort during pregnancy or display any evidence of clinical illness [3] but there is a strong correlation between infection and a documented loss of productivity [20, 21]. Once infected, *N. caninum* persists for the duration of the animal's life [22].

In conclusion, our results indicate that *N. caninum* infection is prevalent in pregnant beef cattle in Phayao. The detection of a high rate of prevalence of *N. caninum* in bovine placenta suggests a high risk of vertical transmission to their foetus; forming a cornerstone of parasite

transmission of the disease in this area. This is the first report by PCR prevalence of *N. caninum* in beef cattle in Thailand. These epidemiological data deserves special attention due to the economic and health impact of this pathogen and could lead to the development of strategies for future prevention and control schemes.

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Table 1 PCR Prevalence of *N. caninum* infection in naturally infected female cattle in Phayao according to the age of cattle.

Age (year)	No. of Animal	No. of positive	Prevalence (%)
2-4	41	13	31.7
≥ 5	33	15	45.5
unknown	22	7	31.8
Total	**96**	**35**	**36.5**

Figures

Fig. 1, PCR detection of *N. caninum* in bovine placenta, Lane M, 100 bp ladder; Nc; *N. caninum* DNA, Tg; *T. gondii* DNA, Dw; distilled water, Lane 2, 3, 4, 7, 8, 11; positive PCR products; Lane 1, 5, 6, 9, 10; negative PCR products.

Fig. 2, Map of the study area in Phayao province, Thailand showing in shade of the prevalence in 7 studied districts,

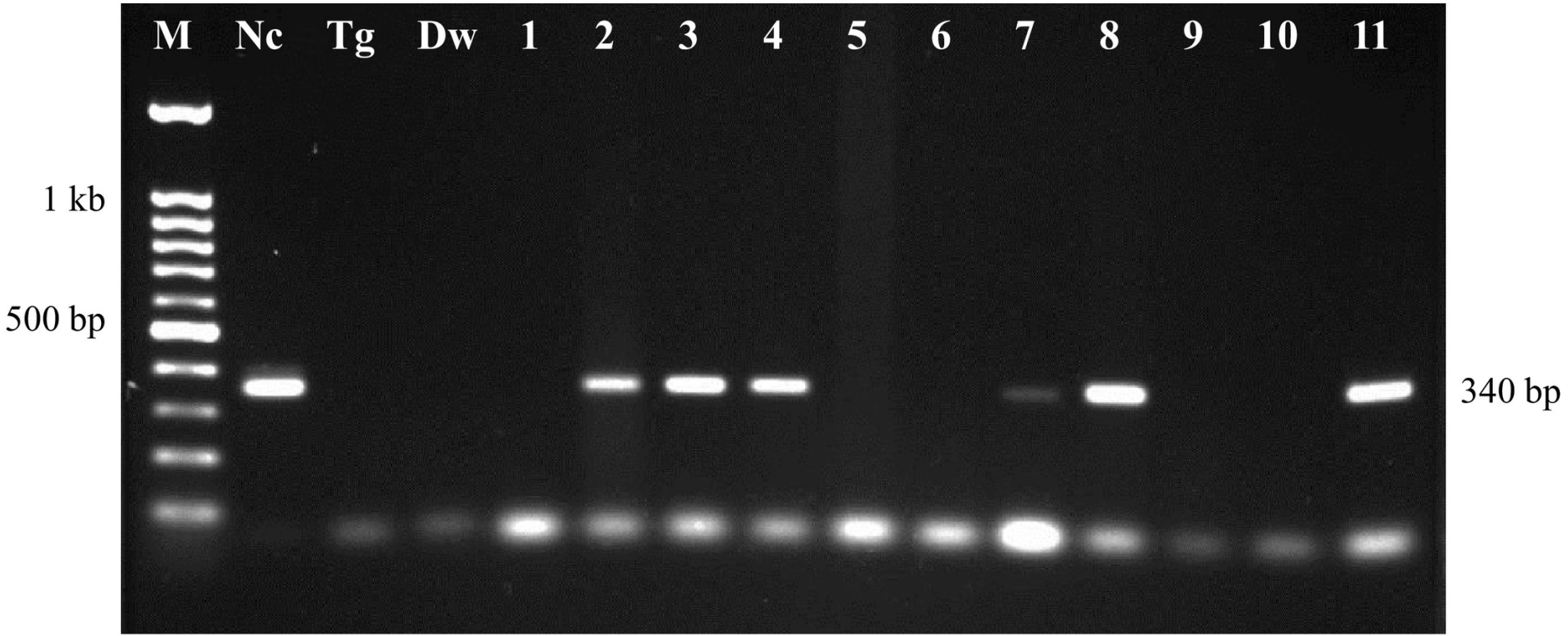

Figure 1

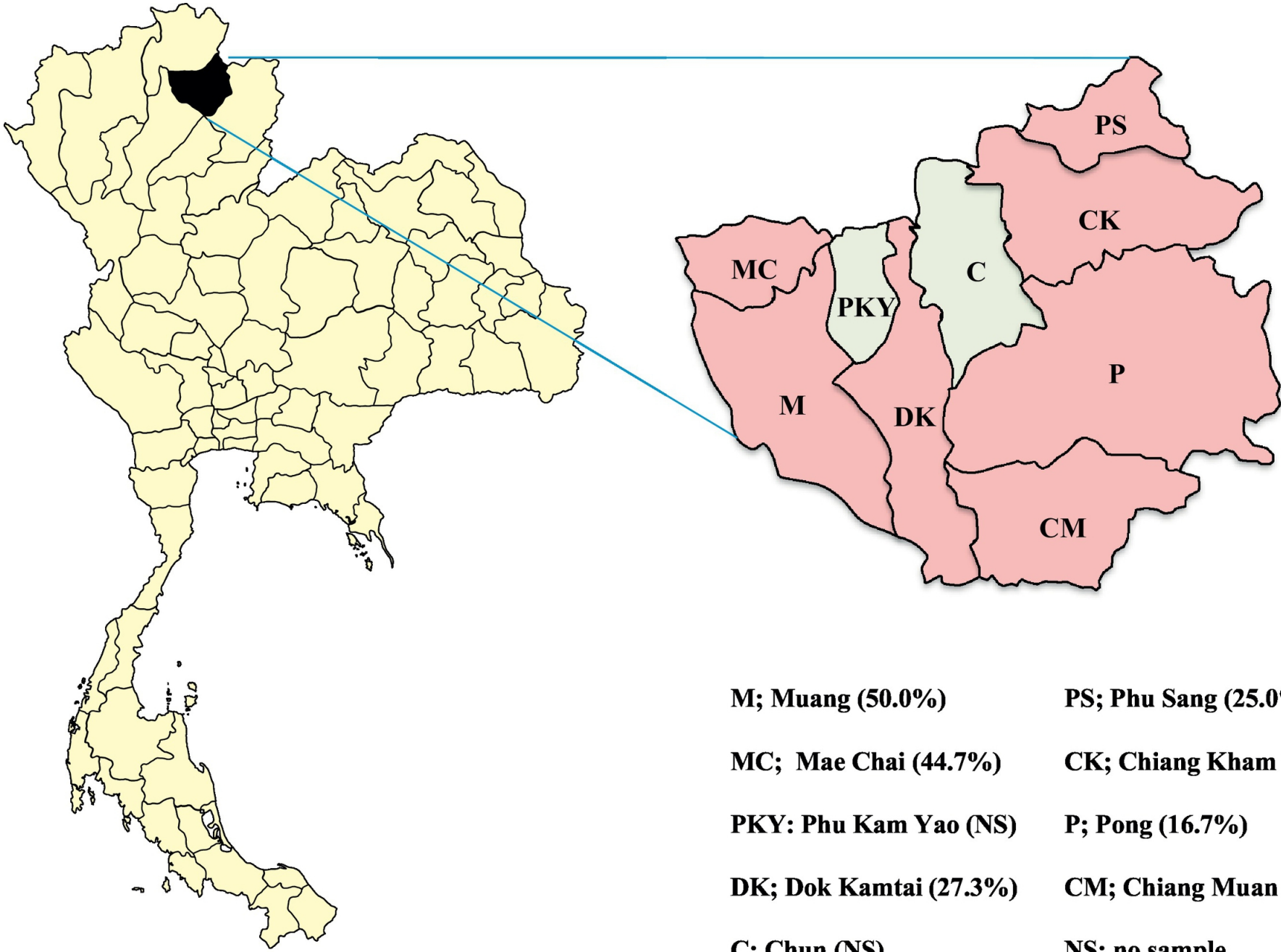
PS
CK
MC
PKY
C
P
M
DK
CM
M; Muang (50.0%)
PS; Phu Sang (25.0%)
MC; Mae Chai (44.7%)
CK; Chiang Kham (30.8%)
PKY: Phu Kam Yao (NS)
P; Pong (16.7%)
DK; Dok Kamtai (27.3%)
CM; Chiang Muan (25.0%)
C; Chun (NS)
NS; no sample

Figure 2